REMARKS

Claims 1-4 are under examination in the present case. Each of these claims is rejected under 35 U.S.C. § 112, first paragraph, and under 35 U.S.C. § 102(b). The rejections are addressed below.

Support for the Amendments

Applicants have amended the specification to conform with the Drawing figure labels.

Support for the claim amendments is found throughout the specification. For example, support for the amendment to claim 1 is found in claim 2 and claim 3 (now cancelled). Support for new claim 12 is found at page 22, lines 2-7; support for new claims 13-15 is found, for example, at Figures 21A and at page 55, lines 22-27. Applicants reserve the right to pursue all canceled subject matter in this, or future, related applications.

Rejections under 35 U.S.C. § 112, first paragraph

Written Description

Claims 1-4 are rejected, under 35 U.S.C. § 112, first paragraph, based on the assertion that the specification fails to provide a written description that conveys to the

skilled artisan that Applicants were in possession of the claimed invention at the time of filing.

Claims 1 and 2 provide methods for compound identification that require expression of a *daf-16* gene in *C. elegans* or in an isolated *C. elegans* cell. Claim 4 specifies that the *daf-16* gene is a nematode gene.

The Office bases the written description rejection of these claims on two grounds: (i) that Applicants have failed to disclose a *daf-16* gene obtained from any organism other than *C. elegans*; and (ii) that Applicants have failed to describe identifying structural or functional characteristics of a *daf-16* gene. This rejection is respectfully traversed.

Disclosure of daf-16 homologs

The Office supports the written description rejection by first asserting that Applicants have failed to disclose any *daf-16* gene other than *C. elegans daf-16*. The Office states:

> The scope of invention as claimed encompasses identification of modulatory compound that decreases the expression or activity of daf-16 gene (obtained from any and all organisms) in any and all nematodes. At best the specification disclosed *C. elegans daf-16* gene...and its function in *C. elegans* worm. *Besides C. elegans daf-16 gene the instant specification fails to disclose daf-16 gene obtained from any other organisms.*

This basis for the rejection is traversed because this statement is incorrect. On this issue, the Examiner's attention is directed to page 55, lines 22-27 of the specification, where Applicants disclose two human homologs of *C. elegans daf-16* that, based on Applicants'

discovery, are expected to function in human insulin signaling. In particular, this portion of the specification states:

> ...human FKHR and AFX genes, identified as oncogene breakpoints but not as insulin signaling genes, are much more closely related to DAF-16 than the next closest relative in either Genbank or in the 94% complete *C. elegans* genome sequence. These data indicate that FKHR and AFX are excellent candidates for subserving the same function as *C. elegans* DAF-16: transduction of insulin signals and convergence with DAF-7-like Smad signals.

Thus, contrary to the Office's assertion, Applicants *have* identified multiple *daf-16* genes. This first basis for the written description rejections should be withdrawn.

Structural and Functional Characteristics of daf-16

In further support of the written description rejection, the Office also states that Applicants have failed to describe identifying structural or functional characteristics of a *daf-16* gene. This assertion is in error and should be withdrawn.

In fact, in satisfaction of the written description requirement, Applicants *have* disclosed characteristic structural and functional features of DAF-16 proteins, as required by M.P.E.P. 2163 II A 3ii, which states:

> The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by ...*disclosure of relevant identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics*, sufficient to show the applicant was in possession of the claimed genus.

These identifying structural and functional characteristics of *daf-16* are detailed below.

Structural Characteristics of daf-16

With respect to structural characteristics of *daf-16*, Applicants direct the Office's attention to Figures 13A and 13B, where Applicants disclose the cDNA sequences of two differentially spliced *daf-16* transcripts, and to Figures 14A and 14B, where Applicants disclose the amino acid sequences of the DAF-16 polypeptide isoforms. Using sequence analysis, Applicants have identified DAF-16 as a member of the forkhead family of transcription factors (page 53, lines 25-27), and, at Figure 21A, Applicants provide an alignment of *C. elegans* DAF-16 with other forkhead family members. This alignment shows that human FKHR and AFX are most closely related to *C. elegans* DAF-16 and share structural characteristics. This alignment also highlights conserved amino acid residues.

Mutational Analysis

The functional importance of amino acid residues conserved among *daf-16* family members is then highlighted by Applicants' mutational analysis (page 53, lines 16-24), which identified three *daf-16* mutations:

> ...(1) a large deletion of conserved regions in *daf-16 (mg ΔF50)* that proves that the *daf-16* null phenotype is a suppression of *daf-2* mutations; (2) an S to L substitution in exon 6 in *daf-16(mg53)* that alters a conserved WKNSIRH motif; and (3) a nonsense mutation in exon 3 in *daf-16(mg54)* that is predicted to truncate one of the daf-16 differentially spliced isoforms (page 53, lines 16-24).

This analysis identified functionally important regions of DAF-16 that are required for wild-type DAF-16 function.

daf-16 Human Homologs

Moreover, Applicants have demonstrated that the structural similarities between the members of the *daf-16* family are echoed in functional relatedness. As stated in Dr. Ruvkun's Declaration, paragraph 1, although *C. elegans* and humans are evolutionarily distant organisms, *C. elegans daf-16* and human proteins FKHR and AFX are highly related. In fact, Dr. Ruvkun and his colleagues have shown that FKHR and DAF-16 are so closely related that the human protein is able to functionally substitute for *C. elegans* DAF-16 *in vivo*. Given this result, those skilled in the art would fully expect that other highly related nematode or mammalian DAF-16 proteins would also substitute for *C. elegans* DAF-16, and that *daf-16* is a member of a family of proteins having shared structural and functional characteristics.

Specifically, Dr. Ruvkun and his colleagues have shown that FKHRL1 and DAF-16 are so closely related that when a *daf-16* human homolog was expressed under the control of the *daf16β* promoter in worms having mutations in *daf-16* and *daf-2*, the human protein was able to replace the worm protein, although the human protein's ability to rescue the *daf-16* phenotype (70%) was somewhat weaker than that of a *C. elegans*

DAF-16 protein (100%) (Declaration of Dr. Ruvkun, paragraph 3, and Lee et al. Curr. Biol. 11:1950-1957, 2001, Exhibit A). These results demonstrate that the human and *C. elegans* proteins are orthologs and indicate that other highly similar DAF-16 family members would also be expected to substitute for *C. elegans* DAF-16.

In addition, as stated in Dr. Ruvkun's Declaration, paragraph 4, in collaboration with Nargis Nasrin and Maria Alexander-Bridges, Dr. Ruvkun has also shown that *C. elegans* DAF-16 functions similarly to human DAF-16 homologs when the *C. elegans* protein is expressed in cultured human hepatocellular carcinoma cells (HepG2 cells), as evidenced by the experiments described in Exhibit B (Nasrin et al., PNAS 97:10412-10417, 2000). In HepG2 cells, DAF-16 and its mammalian homologs, FKHR, FKHRL1, and AFX, activated transcription through the insulin growth factor binding protein (IGFBP)-1-insulin responsive element (IRE). Dr. Ruvkun and his colleagues also found that *C. elegans* DAF-16 and FKHR interacted with both the KIX and E1A/SRC interaction domains of p300/ Creb-binding protein (CBP), as well as the steroid receptor coactivator (SRC). Dr. Ruvkun and his colleagues concluded that DAF-16 and FKHR act as accessory factors to the glucocorticoid response, by recruiting the p300/CBP/SRC coactivator complex to a forkhead factor site in the IGFBP-1 promoter, which allowed the cells to integrate the effects of glucocorticoids and insulin on genes that carry this site. Given this result, it is fully expected that other highly related DAF-16 proteins would function similarly.

In sum, Applicants have shown that *C. elegans daf-16* and human AFX and FKHR proteins are representative of DAF-16 proteins generally. In addition, Applicants have provided a detailed description of such proteins, including the nucleic acid and amino acid sequences of *daf-16*, an alignment of DAF-16 with its most closely related family members, AFX and FKHR polypeptides, identification of the structurally and functionally important domains characteristic of DAF-16 proteins, and a mutational analysis of *C. elegans daf-16*. Moreover, Applicants have shown that human AFX, human FKHR, and *C. elegans daf-16,* as species, are clearly representative of the genus. Applicants have more than satisfied the standards set by the case law; the written description rejection should be withdrawn.

Enablement

Claims 1-4 are further rejected, under 35 U.S.C. § 112, first paragraph, as lacking enablement, based on the assertion that Applicants have failed to enable methods for identifying a compound that modulates any *daf-16* gene other than *C. elegans daf-16* in a nematode or nematode cell other than *C. elegans*. More specifically, the Office bases its rejection on the following grounds: (i) that Applicants failed to disclose a *daf-16* gene from any organism other than *C. elegans*; (ii) that given the low sequence similarity between DAF-16 and FKHR or AFX, it is unpredictable whether FKHR or AFX could substitute for endogenous *daf-16* in any and all nematodes or isolated nematode cells; (iii)

that the phenotype of a transgenic nematode expressing an exogenous transgene is unpredictable; and (iv) that undue experimentation would be required to carry out the claimed method in any nematode other than *C. elegans* using any *daf-16* gene other than *C. elegans daf-16*.

As an initial matter, Applicants note that claims 1, 2, and 4 now provide methods for identifying compounds that decrease *daf-16* expression or activity in a *C. elegans* or *C. elegans* cell. Claim 3 has been cancelled.

daf-16 Homologs

As detailed above, contrary to the Office's assertions, Applicants have in fact identified two human *daf-16* homologs, AFX and FKHR. Additional *daf-16* genes could be identified using methods detailed in Applicants' specification. For example, at pages 76 and 77, Applicants disclose 5 amino acid sequences that may be used to identify a *daf-16* gene present in a sequence database or that may be used to design degenerate probes to identify a *daf-16* gene present in a genomic or cDNA library. Provided with these specific sequences, the skilled artisan could easily identify virtually any *daf-16* nucleic acid sequence using no more than routine methods described in Applicants' specification, for example, at pages 77-79.

Predictability of C. elegans Transgenic Phenotype

The Office asserts that given the low sequence similarity that exists between FKHR and AFX, it is unpredictable whether such proteins could functionally substitute for *daf-16* in *C. elegans*. The Office supports this assertion citing Ngo et al. (The Protein Folding Problem and Tertiary Structure Prediction, Merz and Le Grand, editors, Birkhauser, Boston, MA, pp. 433, and 492-495, 1994) and Rudinger (Peptide Hormones, Parsons, editor, University Park Press, Baltimore, MD, pp. 1-7, 1976). Ngo et al. teach that at present the structure of a protein cannot be predicted using a computer algorithm. Rudinger teaches that the significance of particular amino acids and sequences must be determined experimentally.

As applied to *daf-16* and its human homologs, specifically, this general concern is unwarranted. As indicated above, Dr. Ruvkun and his colleagues have demonstrated that DAF-16 and its human homologs are functionally interchangeable, making predictions using computer algorithms unnecessary.

The Office further asserts that the phenotype of a transgenic *C. elegans* expressing an exogenous *daf-16* transgene is unpredictable, and cites Wood et al. (*Comparative Medicine*, 50:12-15, 2000, "Wood") and Sigmund et al. (Arterioscler. Thromb. Vasc. Biol. 20: 1425-1429, 2000) in support of this position. Turning first to Wood, Wood teaches that specific paradigms are useful in assessing the phenotype of a *genetically modified rodent* (page 12, left column, first paragraph).

> The purpose of this paper is to describe a general scheme of approaching the overall phenotype assessment of the large number of genetically altered or spontaneous *mutant mice*, as well as other *rodent models* currently being developed.

The claimed invention features screening methods that require *transgenic nematodes*. The claimed invention does not require the production of a *genetically modified rodent* and the issues raised in Wood are therefore of little or no relevance.

Turning next to Sigmund, Sigmund teaches that the effects of genetic variability in *mutant mouse models* can be minimized by using strategies to minimize genetic variation between experimental and control mice. These strategies include the use of isogenic strains or congenic strains of mice, and successive back-crossing of genetically altered mice (page 1426, right column, second paragraph, to page 1428, left column, first paragraph), as well as the evaluation of large numbers of mice to reduce variability associated with epigenetic effects.

Once again, Applicants note that the claimed invention is directed to *nematodes*, not mice. Moreover, the methods taught by Sigmund for minimizing genetic variability between experimental and control mice, i.e., the use of isogenic strains are routine in nematode biology as noted in Dr. Ruvkun's Declaration, at paragraph 6.

In addition, as evidenced in Exhibit A, Applicants have demonstrated that the human homologs of the nematode DAF-16 protein, FKHR and AFX, are able to *functionally substitute* for *C. elegans* DAF-16 *in vivo*. In view of this experiment,

predictability is not an issue, and this basis for the enablement rejection should be withdrawn.

Quantity of Experimentation

Finally, the Office asserts that undue experimentation would be required to practice the claimed methods. Specifically, the Office states:

> ...considering the unpredictability in the art and the limited guidance provided in the specification as filed one skill in the art would have to engage in excessive and undue amount of experimentation to exercise the invention as claimed. The undue experimentation required would include making and testing any and all nematodes or genetically engineered nematode cells encoding any *daf-16* like genes obtained from any and all organisms.

Applicants disagree. In fact, the specification provides ample guidance to carry out the invention as claimed. For example, Applicants teach methods for producing transgenic *C. elegans* at pages 65-66; methods for identifying additional *daf-16* genes are described at pages 74-80, under the headings "Cloning Mammalian Daf Sequences;" screening methods for identifying modulatory compounds at pages 85-91, under the headings, "Screening Systems for Identifying Therapeutics," and "Test Extracts and Compounds;" and methods for evaluating the effects of a modulatory compound on *C. elegans* at pages 92-93, under the heading, "*C. elegans*." Thus, using no more than routine methods described in Applicants' specification, the skilled artisan could easily identify those compounds that modulate *daf-16* expression. Contrary to the Office's assertion, such

routine screening does not constitute undue experimentation.

In analyzing what constitutes undue experimentation, the MPEP (§ 2164.06) cites *In re Wands*, (858 F.2d 731, 8 USPQ2d 1400 (Fed Cir. 1988)):

> The test is not merely quantitative, since *a considerable amount of experimentation is permissible, if it is merely routine*, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. (emphasis added)

At the time of filing, a skilled artisan could easily identify compounds that modulate *daf-16* expression using methods described in Applicants' specification. Such screening could easily be accomplished using standard techniques; no undue experimentation is required. For all of the above reasons, the enablement rejection should be withdrawn.

Rejection under 35 U.S.C. § 102(b)

Claims 1-4 are further rejected under 35 U.S.C. § 102(b) based on the assertion that the claims are anticipated by Gottlieb et al. (Genetics 137:107-120, 1994, hereafter "Gottlieb"). This rejection is respectfully traversed.

The pending claims feature methods for identifying compounds that modulate *daf-16* expression or activity. The Office states "[t]he cited art clearly teaches a method that can identify a compound that is capable of decreasing the expression or activity of *daf-16* gene." This statement is in error.

Gottlieb neither teaches nor suggests any method of compound identification.

Gottlieb teaches that, under conditions of high population density (high pheromone, low food), developing *C. elegans* form dauer larvae, and that dauer formation is controlled by genetic interactions among *daf* genes as determined by genetic epistasis analysis. Gottlieb characterizes her results as follows:

> When animals are exposed to growth-promoting conditions, defined as high food and low pheromone, the sensory neurons send a signal which prevents dauer formation through the inactivation, either directly or indirectly, of the Daf-d gene *daf-12* and activation of the Daf-c genes *daf-2* and *daf-23*. Under these conditions, *daf-2* and *daf-23* would function to prevent dauer formation by the inactivation of the Daf-d gene *daf-16* as well as by negatively regulating the activity of *daf-12*...When exposed to high pheromone dauer-inducing conditions, the sensory neurons no longer send a growth-promoting signal. This leads to the activation of *daf-12* and the inactivation of *daf-2* and *daf-23*. The absence of *daf-2* and *daf-23* function relieves repression of *daf-16*. The resulting *daf-16* gene activity represses non-dauer development and/ or activates dauer entry and prevents dauer recovery (page 117, right column, first paragraph, to page 118).

Thus, Gottlieb teaches a genetic pathway that controls dauer formation. Gottlieb fails to teach or suggest compound screening methods, and therefore fails to anticipate the claimed invention.

Regarding the Office's statement that the dauer larvae has a "thin body" and is "non-obese," Applicants note that Gottlieb fails to draw any connection between the dauer pathway and obesity. Instead, Gottlieb teaches that the dauer larvae's morphological changes promote resistance to chemical treatments and desiccation. Gottlieb states:

> ...in environments with a high density of animals and a corresponding high level of dauer-inducing pheromone, animals arrest development following the second larval molt as specialized dauer larvae. The formation of a dauer larvae involves

> morphological changes in many tissues of the animal: dauer larvae are thinner...have a specialized cuticle, a pharynx that is remodeled and plugged and intestinal cells that appear dark. In addition, the molting cycle is suppressed and all feeding and growth are arrested. Because of these adaptations, the animals are more resistant to harsh chemical treatments and desiccation (p. 107, left column, first paragraph).

Regarding the Office's statement that Gottlieb teaches "the construction of worms encoding *daf-16* wild-type or mutated *daf-16* transgenes (citations omitted)," Applicants point out that this is also incorrect. Applicants note that Gottlieb fails to identify any *daf-16* nucleic acid sequence, and thus could not and did not produce a worm expressing any *daf-16* transgene. Indeed, Applicants were the first to identify and characterize *daf-16* nucleic acid and amino acid sequences, and the present invention is based on this novel discovery. The passages cited by the Office describe genetic interactions among *daf* genes (page 108, left column, paragraph 2), describe dauer formation in various *C. elegans* dauer defective mutants (page 109, column 1, paragraphs 1-4), and describe the *genetic* construction of *C. elegans* strains having multiple mutations in *daf* genes (pages 108-109). However, Gottlieb does not isolate a *daf-16* transgene and cannot therefore describe the construction of a worm having such a transgene.

In sum, Gottlieb fails to teach a method for identifying candidate modulatory compounds that decrease the expression or activity of DAF-16 using a *C. elegans* or *C. elegans* cell expressing a *daf-16* gene. Gottlieb therefore fails to teach important elements of claims 1-4, and the anticipation rejection should be withdrawn.

CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is respectfully requested.

If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

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Exhibit A

1950 Brief Communication

R gulation of *C. elegans* DAF-16 and its human ortholog FKHRL1 by the *daf-2* insulin-like signaling pathway

Raymond Y.N. Lee*, Jürgen Hench† and Gary Ruvkun

C. elegans insulin-like signaling regulates metabolism, development, and life span. This signaling pathway negatively regulates the activity of the forkhead transcription factor DAF-16. _daf-16_ encodes multiple isoforms that are expressed in distinct tissue types and are probable orthologs of human FKHRL1, FKHR, and AFX. We show that human FKHRL1 can partially replace DAF-16, proving the orthology. In mammalian cells, insulin and insulin-like growth factor signaling activate AKT/PKB kinase to negatively regulate the nuclear localization of DAF-16 homologs (reviewed in [1]). We show that the absence of AKT consensus sites on DAF-16 is sufficient to cause dauer arrest in _daf-2(+)_ animals, proving that _daf-16_ is the major output of insulin signaling in _C. elegans_. FKHR, FKRHL1, and AFX may similarly be the major outputs of mammalian insulin signaling. _daf-2_ insulin signaling, via AKT kinases, negatively regulates DAF-16 by controlling its nuclear localization. Surprisingly, we find that _daf-7_ TGF-β signaling also regulates DAF-16 nuclear localization specifically at the time when the animal makes the commitment between diapause and reproductive development. _daf-16_ function is supported by the combined action of two distinct promoter/enhancer elements, whereas the coding sequences of two major DAF-16 isoforms are interchangeable. Together, these observations suggest that the combined effects of transcriptional and posttranslational regulation of _daf-16_ transduce insulin-like signals in _C. elegans_ and perhaps more generally.

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Results and discussion

Insulin signaling in *C. elegans* regulates the developmental decision to grow reproductively or arrest at the dauer stage, and it affects metabolism, stress resistance, fertility, and life span. Because strong loss-of-function mutations in *daf-16* can suppress all of the phenotypes caused by mutations in *daf-2* insulin/IGF receptor tyrosine kinase [2], temporally and spatially regulated expression of *daf-16* may be important to diversify the biological outputs of insulin signaling. The *daf-16* locus encodes three transcripts: a1, a2, and b, generated by alternative splicing and probable alternative promoters [3]. The predicted protein products of *daf-16a1* (DAF-16A1) and *daf-16a2* (DAF-16A2) are closely related, differing by only two amino acids, which is due to alternative splicing in exon 3. In contrast, *daf-16a* (refer to *a1* and *a2* together) and *daf-16b* share the C-terminal 319 amino acids encoded by common exons but differ in their amino-terminal coding regions: upstream from the common domain, DAF-16A1 (191 residues encoded by exon 1–4) and DAF-16B (211 residues encoded by exon 5) are only 32% identical. The 5′ ends of the *daf-16a* and *daf-16b* transcripts are separated by 8.4 kb, strongly supporting distinct promoters and possibly distinct enhancers for each transcript. A 6.4-kb segment from 5′ to *daf-16a* directs expression in body wall muscles, hypodermis, and intestine [3], whereas a 6.1-kb segment from 5′ to *daf-16b* directs expression in the pharynx (S. Ogg and G.R., unpublished data). Thus, there may be distinct *cis*-regulatory regions that control the expression of each isoform. Because of their distinct expression patterns and N-terminal coding sequences, *daf-16a* and *daf-16b* could mediate distinct functions.

We tested this possibility by analyzing the phenotype of *daf-16(m26)* and *daf-16(mg54)*, predicted to specifically affect *daf-16a* [3, 4]. *daf-16(m26)* carries a mutation in the splice donor site of intron 2, whereas *daf-16(mg54)* carries a nonsense mutation in exon 3, both of which are specific to *daf-16a*. We tested the ability of these *daf-16a*-specific mutants to form dauers constitutively (dauer arrest constitutive) or to live long as adults (ageing alteration) in a *daf-2(e1370)* mutant background. When *daf-16* is wild-type, *daf-2(e1370)* animals are nearly 100% dauer arrest constitutive at 25°C [5]. When animals of this genotype are grown at 15°C until the L4 stage and are then shifted to 25°C, the adults have an average life span of 30 days, about twice that of wild-type ([6] and Figure 1). The *daf-16(mgDf47)* null mutation, which deletes exon 5 through the end of the coding region and therefore lacks the DNA binding domains of both DAF-16A and DAF-16B [3], fully suppresses both the dauer arrest and longevity phe-

Table 1

Dauer arrest phenotype of *daf-16(x); daf-2(e1370)* mutants.

X =	Reproductive development	Dauer(-like) arrest	Other
mgDf47	95.5% (734)	0.1% (1)	3.4% (26)
mg54	92.3% (515)	0.2% (1)	7.5% (42)
m26	96.0% (1099)	2.1% (24)	1.9% (21)
+	0%	75.6% (163)	24.5% (53)

Synchronized (within 3 hr) progeny of gravid mutant mothers were shifted to the test temperature (25°C) for 46–52 hr. They were then scored for the dauer arrest constitutive phenotype visually. For all tables, reproductively developing animals included L4 and adult animals. Dauer(-like) arrested animals were either complete or partial dauers (showing one or more features, such as radial constriction, dauer alea, and pharyngeal constriction) or L2d animals. "Other" includes animals that were of L1, L2, or L3 stages that had no dauer features. The number of animals is in parentheses. Distributions of arrested animals between the "dauer(-like)" and the "other" classes were somewhat variable from experiment to experiment. However, the combined percentage of the two classes was quantitatively reproducible for a given genotype. Strains used were: GR1309 for *daf-16(mgDf47); daf-2(e1370)*, GR1308 for *daf-16(mg54); daf-2(e1370)*, GR1117 for *daf-16(m26); daf-2(e1370)*, and GR1122 for *daf-2(e1370)*.

notypes of a *daf-2* mutant: *daf-16(mgDf47); daf-2(e1370)* double mutant animals form virtually no dauers under replete conditions at 25°C, and the adults live even shorter than wild-type ([5]; Table 1 and Figure 1). Under similar conditions, few *daf-16(mg54); daf-2(e1370)* animals (1/558 assayed) or *daf-16(m26); daf-2(e1370)* animals (24/1131 assayed) arrest at the dauer stage (Table 1). Similarly, neither *daf-16(m26); daf-2(e1370)* nor *daf-16(mg54); daf-2(e1370)* animals lived longer than wild-type control animals (Figure 1). Thus, *daf-16a*-specific mutations behave similarly to *daf-16(null)* in their ability to suppress *daf-2(e1370)*. These results indicate that the *daf-16b* activity that remains in these *daf-16a* probable null mutants is not sufficient to supply *daf-16* gene function.

The ability of a *daf-16a* mutation to fully suppress both the aging and dauer arrest phenotypes of *daf-2* strongly suggests that *daf-16a* is a major target of *C. elegans* insulin signaling. Alternatively, animals depend on the function of both *daf-16a* and *daf-16b*; the loss of either one may be sufficient to abrogate *daf-16* function. There is no *daf-16b*-specific lesion in our large collection of *daf-16* mutant alleles (in addition to previously published data in [3] and [4], we sequenced exon 5 in 12 mutants; data not shown), weakly supporting a model that *daf-16b* has a less-central function in dauer arrest than *daf-16a*.

We addressed the function of *daf-16b* using RNA interference (RNAi; [7]). The *daf-16a* and *daf-16b* exons that encode the distinct N-terminal regions are divergent enough that double-stranded RNA corresponding to *daf-16b* will not also target *daf-16a* (the longest uninterrupted run of nucleic acid identity is 5 base pairs between *daf-16b*-specific exon 5 used in RNAi and the entire

Figure 1

Adult life span of *daf-16; daf-2* double mutants. The life span of adult animals grown at 25°C presented as a Kaplan-Meier plot. *mgDf47* is a null allele of *daf-16*, whereas *mg54* and *m26* are alleles that specifically affect the *daf-16a* isoform [3]. Strains used were: Bristol N2 for wild-type (n = 592), GR1309 for *daf-16(mgDf47); daf-2(e1370)* (n = 99), GR1308 for *daf-16(mg54); daf-2(e1370)* (n = 104), GR1117 for *daf-16(m26); daf-2(e1370)* (n = 99), GR1122 for *daf-2(e1370)* (n = 40), and GR1329 for *daf-16(mgDf47)* (n = 229). The asterisk indicates that the *daf-2(e1370)* life span was determined in slightly different conditions than the rest, on regular rather than FUdR-containing (fluorodeoxyuridine) plates. FUdR does not affect the life span of animals [20].

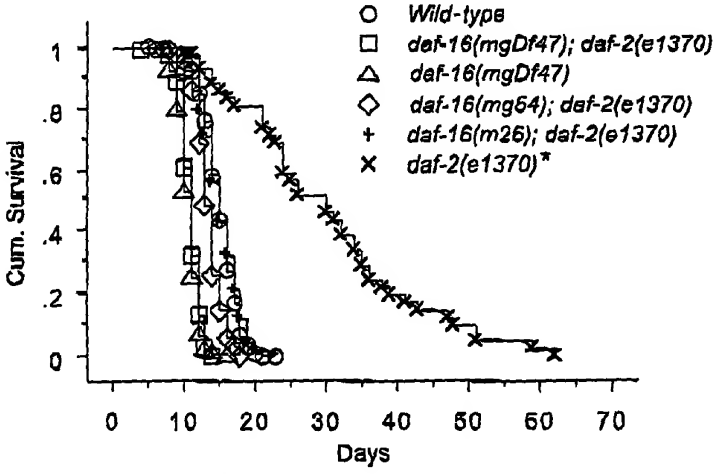


Figure 2

GFP expression driven by a *daf-16*β enhancer/promoter element. Fluorescent micrographs of *daf-16(mgDf47)* animals that carry a *daf-16*-rescuing translational GFP fusion *Ex[daf-16*β*::GFP::DAF-16B]* transgene. **(a)** An L1 animal showing GFP expression in neurons; four fluorescent neurons are marked by arrowheads. **(b)** A late L4 animal. GFP was expressed in the pharynx (marked by triangles), somatic gonad (with an arrow pointing to the vulva), and in neurons in the tail (marked by an arrowhead). Transgene array *Ex[daf-16*β*::GFP::DAF-16B]* was made as a complex extrachromosomal array in order to get better stability of transgene expression [21]. GR1329 *daf-16(mgDf47)* animals were transformed with a mixture of PvuII-digested worm genomic DNA (100 μg/ml) and *daf-16*β*::GFP::DAF-16B* minigene (0.25 μg/ml, in the form of PCR products). Higher concentrations of *daf-16*β*::GFP::DAF-16B* drastically reduced the viability of transgenic embryos (data not shown). DNA templates used: R13H8, a genomic cosmid clone from A. Coulson, and pPD117.01, a GFP vector. PCR primer sequences and procedures for fusions are available at http://whitney.caltech.edu/~raymond/daf16.html or upon request.

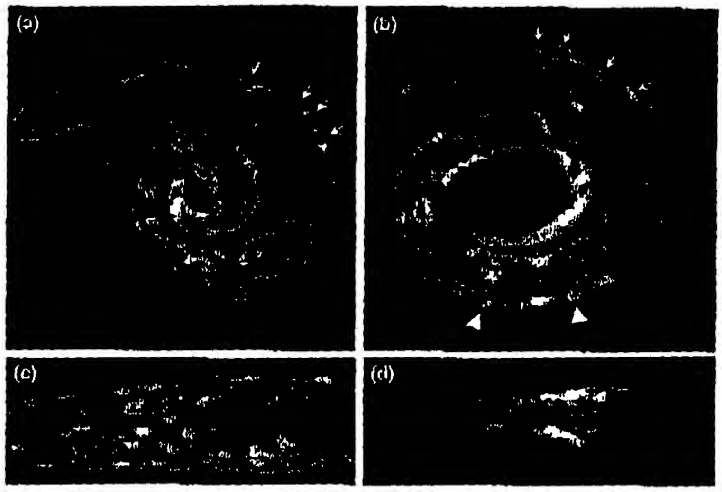
daf-16a1 transcript). RNAi experiments showed that inactivation of *daf-16b* in *daf-2(e1370)* had much weaker effects on dauer arrest than did the RNAi inactivation of *daf-16a* alone: progeny of *daf-2(e1370)* animals that received *daf-16b* dsRNA were 100% dauer arrest constitutive (44 of 44 animals), whereas progeny from mothers that received *daf-16a* dsRNA (corresponding to exons 1–4) were 0% dauer arrest constitutive (0 of 69 animals). The combination of *daf-16a*- and *daf-16b*-specific dsRNA was also effective in suppressing *daf-2(e1370)* dauer arrest (1 of 75 progeny became a dauer; 74 developed reproductively). These results showed that *daf-16a* is the major genetic activity from the *daf-16* locus for *daf-2*-mediated dauer arrest and longevity control.

What makes *daf-16a* functionally different from *daf-16b*? We first considered the possibility that the two isoforms are differentially expressed. By transgenic rescue experiments, we found that a 2-kb (12,727–14,813 bp in cosmid R13H8, accession number AF039717) genomic region immediately 5′ to the predicted first ATG codon of *daf-16b* was sufficient to direct expression of *daf-16b*-coding sequences to rescue the dauer arrest-defective phenotype of *daf-16(mgDf47)* ([3] and see below). We named this transcriptional regulatory element *daf-16*β. We analyzed the cellular expression pattern of a *daf-16*β-promoter::GFP::DAF-16B fusion gene, in which GFP was fused at the less well-conserved N terminus of DAF-16B. This fusion gene rescues the dauer arrest defective phenotype of *daf-16(mgDf47)* (see below). We first detected the functional GFP::DAF-16B fusion protein fluorescence in early embryos, prior to morphogenesis. After hatching and in all later developmental stages, transgenic larvae showed GFP in the pharynx and in many neurons throughout the body. From the late L3 stage onward, GFP was also detected in somatic gonads (Figure 2).

The *daf-16*α promoter element was defined by the 6.4-kb (48–6408 bp in cosmid R13H8) genomic region 5′ to the initiation ATG of *daf-16a*. We analyzed the expression pattern of a *daf-16*α-promoter::GFP::DAF-16B fusion gene, which similarly rescues the dauer arrest-defective phenotype of *daf-16(mgDf47)* (data not shown). GFP was first detected in comma-stage embryos. After hatching, transgenic animals showed high levels of GFP expression in almost all somatic cells. In contrast to the *daf-16*β fusion gene, little GFP was detected in somatic gonad or pharynx (except occasionally in one or two unidentified cells in the terminal bulb; Figure 3 and data not shown).

In order to validate the significance of these expression patterns, we tested these fusion genes bearing full DAF-16B protein-coding segments for the rescue of different aspects of the *daf-16* mutant phenotype. There are multiple features of dauer larvae that differ from reproductively developing L3 larvae: a constricted pharynx, a shift to fat storage, cuticular structures called dauer alae, as well as resistance to stress and an increased life span. We found that each of *daf-16*α and *daf-16*β promoter/enhancer DNA segments can direct expression of DAF-16 proteins to rescue some aspects of *daf-16* loss-of-function mutant phenotype, but their activities were partially complementary to each other (summarized in Table 2). *daf-16(mgDf47)* animals are partially dauer defective [8], unable to form complete, SDS-resistant dauers under inducing conditions (such as on starved plates), and they have a shorter life span than wild-type ([5]; Figure 1 and data not shown). We found that *daf-16(mgDf47)* transgenic animals bearing either *daf-16a*::DAF-16A1 or *daf-16*β::DAF-16B were able to form SDS-resistant dauer larvae on starved plates. However, animals carrying *daf-16a*::DAF-16A1 formed dauers with little pharynx remodeling, which is normally found in dauer animals (0

Figure 3



DAF-16 protein nuclear localization is dynamically regulated by *daf-2* and *daf-7*. Fluorescent micrographs of animals carrying *Ex[daf-16α::GFP::DAF-16B]*, a *daf-16/GFP* fusion gene that can rescue *daf-16* mutant dauer-defective phenotype (data not shown). **(a)** An L1 animal of genotype *daf-16(mgDf47); daf-2(+)*. GFP::DAF-16 is mostly cytoplasmic with a perinuclear concentration. The soma of four neurons in the head are marked by arrows. **(b)** An L1 animal of genotype *daf-16(mgDf47); daf-2(e1370)*. GFP::DAF-16 is prominently nuclear localized. The nuclei of four neurons in the head and two hypodermal cells in the midbody are marked by arrows and triangles, respectively. **(c)** The head region of a *daf-16(mgDf47); daf-7(m62)* L2d predauer-stage animal. GFP::DAF-16 was almost exclusively localized in nuclei throughout the animal. **(d)** The head region of a *daf-16(mgDf47); daf-7(m62)* dauer animal carrying the transgene showing a more diffuse, primarily perinuclear GFP::DAF-16 localization. *daf-16α*-promoter::GFP::DAF-16B fusion PCR product (concentration at 2.5 ng/μl) was mixed with 98 ng/μl pRF4 [14] *rol-6* DNA and was used to transform GR1329 *daf-16(mgDf47)* animals. The stably transmitting transgene was then crossed into *daf-16(mgDf47); daf-2(e1370)* and *daf-16(mgDf47); daf-7(m62)* mutants.

of 30 SDS-resistant dauers examined had a constricted pharynx), whereas dauers that carried *daf-16β*::DAF-16B had more complete pharyngeal constriction (11 of 17 dauers examined). This result is consistent with an autonomous DAF-16 protein function in the pharynx, because the *daf-16β* promoter element is active in the pharynx, whereas *daf-16α* is not.

We also found a difference between the life span regulatory activity of the two fusion genes. *daf-16(mgDf47); Ex[daf-16α*::DAF-16A1] transgenic animals had an average adult life span 65% longer that of control *daf-16(mgDf47)* animals, whereas *Ex[daf-16β*::DAF-16B] transgenic animals lived, on the average, only 14% longer than the control (Table 2).

Table 2

Comparisons of *daf-16α* and *-β* promoter-driven fusion gene activity.

Transgene	Phenotype in *daf-16(mgDf47)*		
	SDS-r	Pha const.	Mean lifespan (N)
Marker only	0/3 lines	n/a	78% (40), 96% (39)[a]
daf-16α::DAF-16A1	2/3 lines	0/30 dauers	165% (40)
daf-16α::DAF-16B	2/3 lines	0/29 dauers	165% (40)
daf-16β::DAF-16A1	3/3 lines	15/25 dauers	114% (40)
daf-16β::DAF-16B	2/2 lines	11/17 dauers	114% (40)

SDS-r: presence of 1% SDS-resistant dauers on starved plates. Each line is an independently generated transgenic strain. Pha const.: pharyngeal constriction in SDS-resistant dauers. Lifespan: mean adult lifespan measured at 25°C, compared with *daf-16(mgDf47)* without transgene.

[a]Two independent lines showing different population lifespans.

daf-16α-promoter::DAF-16A1, *daf-16α*-promoter::DAF-16B, *daf-16β*-promoter::DAF-16A1, and *daf-16β*-promoter::DAF-16B fusion PCR products (concentration at 2.5 ng/μl) were each mixed with 50 ng/μl pRF4 and 50 ng/μl pTG96 (expresses GFP in every nucleus, except, germ line [22]) plasmid DNA and were used to transform GR1329 *daf-16(mgDf47)* animals. Transgenic lines were also made with markers only to serve as controls. DNA templates used in making PCR fusions: R13H8, a genomic cosmid clone from A. Coulson; pdaf16a1, a *daf-16a1* cDNA clone described previously [3]; pdaf16b, a cDNA clone isolated in this study that contains the ORF part of AF020344 [3].

Given the fact that DAF-16A and DAF-16B proteins have very different sequences at their N termini, we tested if the differences in coding sequences contribute to the difference in function of the fusion genes. By swapping the protein-coding segments between the genes to generate *daf-16α*::DAF-16B and *daf-16β*::DAF-16A1 fusion transgenes, we found that the protein-coding sequences were essentially interchangeable for life span and dauer arrest regulation; no significant differences were detected when comparing fusion genes with the same promoter element but different coding sequences (Table 2). Thus, at least in the context of these fusion genes, transcriptional rather than protein sequence differences subserve the particular biological functions of *daf-16*.

Our genetic and fusion gene analyses indicate that *daf-16a* encodes the most important protein products of the *daf-16* locus and suggest that the distinction between the *daf-16a* and *daf-16b* gene activities is likely based on their distinct expression domains rather than the differences between the DAF-16A and DAF-16B proteins (Figure 1 and Table 2). Although we did not compare the expression levels of the *daf-16* promoter fusion genes, the fusion genes were injected at similar low-copy injection concentrations (2.5 ng/μl), and multiple transgenic lines were tested. We therefore favor the model that the *daf-16α* promoter activity controls aging, whereas the *daf-16β* promoter activity is necessary for pharyngeal restructuring during dauer arrest. Both promoters contribute to dauer arrest. It may be that *daf-16α* and *daf-16β* promoters supply combined trascriptional regulation more to *daf-16a* than to *daf-16b* to cause dauer arrest and long life span. However, our transgenic analyses may have exaggerated the role of *daf-16β* promoter in dauer formation; chromosomal expression of *daf-16b* alone may not be sufficiently high to significantly effect dauer formation. Furthermore, we cannot rule out that expression of *daf-16b* is also affected in *daf-16(mg54)* and *daf-16(m26)* mutants. Further quantitative analyses of expressed RNA species in different genetic backgrounds will resolve these issues.

Mammalian AFX, FKHR, and FKHRL1 are closely related in sequence to worm DAF-16. These mammalian genes are expressed in distinct tissue types and have been implicated in insulin and insulin-like growth factor signaling pathways (reviewed in [1]). To prove the orthology between *daf-16* and these related genes, we tested the ability of human FKHRL1 to substitute for DAF-16 in the functional fusion genes described above. We found that a *daf-16β*::FKHRL1 fusion gene supplies *daf-16* gene activity to a *daf-16(mgDf47); daf-2(e1370)* double mutant (Table 3). Under restrictive conditions, animals that carried a *daf-16β*::FKHRL1 fusion gene showed significantly higher levels (>70%) of *daf-2* mutant-like dauer and early larval arrest compared to the nontransgenic controls (3%). Under similar conditions (except at lower injection concentrations), the *daf-16β*::DAF-16B fusion gene showed 100% arrest. However, most arrested animals bearing *daf-16β*::FKHRL1 showed no or partial dauer features, such as body radial constrictions, dauer alae, and pharyngeal constrictions. We conclude that human FKHRL1 can function like DAF-16 in mediating insulin signaling in *C. elegans*, but that its activity is weak.

Mammalian FKHRL1 is negatively regulated by AKT/PKB kinases [9]. *C. elegans akt-1* and *akt-2* have been shown genetically to negatively regulate *daf-16* [10]. On DAF-16 (either the A or B form), there are four sites that conform to the consensus of mammalian AKT phosphorylation. To address the biological importance of AKT phosphorylation, we assayed the phenotypic effects of expressing the AKT phosphorylation-defective DAF-16A1-4A mutant [11] in the *daf-16(mgDf47)* background. In the anticipation that transgenic animals might arrest constitutively as dauers, we isolated the transgenic animals after microinjection by feeding the injected parents and offspring on *Escherichia coli* that express *daf-16* dsRNA to keep the DAF-16 expression levels low as lines were established [12]. Phenotype assays were then done on the decendents of those animals now feeding on non-*daf-16* RNAi *E. coli* for one generation or two.

We found that *daf-16(mgDf47)* animals bearing the *daf-16α*::DAF16A1-4A fusion gene showed moderate (~60%) to nearly complete (99%) constitutive dauer or otherwise larval arrest under nondauer-inducing conditions, depending on whether they were the first or second generation progeny of animals that were fed *daf-16* dsRNA-expressing bacteria (Table 4). This result indicates that DAF-16 phosphorylation on some or all four S/T residues by AKT (or other related) kinases is a crucial aspect of *daf-16* gene function and a pivotal point of regulation. We could not measure the adult life span of these transgenic animals because the variably penetrant arrest phenotype interfered with the isolation of a synchronized adult population. Because decrease in *akt-1* and *akt-2* gene activity causes a similar phenotype to the inactivation of AKT phosphorylation sites on DAF-16 [10], we favor the model that AKT-1 and AKT-2 are the major inputs of DAF-16 at these sites.

In contrast to our findings, Lin et al. reported that a *daf-16A-4A* mutant expressed from a transgene rescued a *daf-16*(null) mutant but did not cause any constitutive dauer arrest or longevity phenotype in a *daf-2(+)* genetic background. They concluded that *daf-2* regulates *daf-16* via a non-AKT consensus site-based mechanism [13]. We consider three possibilities to reconcile the difference between their and our observations. First, the expression levels of the transgenes may be significantly different. Since they did not use RNAi to inhibit any dauer arrest phenotypes in the initial lin generation by transforma-

Table 3

Rescue of the *daf-16(mgDf47); daf-2(e1370)* dauer-defective phenotype by human FKHRL1.

Transgene	Reproductive development (N)	Dauer(-like) arrest (N)	L3/L2 (N)	L1/egg (N)
No transgene control	97.2% (141)	0.7% (1)	1.4% (2)	0.7% (1)
daf-16β::DAF-16B	0%	48% (12)	20% (5)	32% (8)
daf-16β::FKHRL1(line #1)	29.0% (31)	31.8% (34)	1.9% (2)	37.4% (40)
daf-16β::FKHRL1(line #2)	9.5% (6)	47.6% (40)	17.9% (15)	25.0% (21)

Animals were scored 51 hr after egglay, at 25°C. The PCR product of *daf-16β-promoter*::FKHRL1 (10 ng/μl) was mixed with pRF4 (45 ng/μl) and pTG96 (45 ng/μl) and was used to transform GR1909 *daf-16(mgDf47); daf-2(e1370) animals*. A template used in the PCR involving human FKHRL1 was pHa-FKHRL1-HA, a HA-tagged human FKHRL1 cDNA plasmid, a gift from M.J. Anderson and K.C. Arden (AF032886, [23]).

tion, it is possible that their transgenes were selected to express at lower levels. Indeed they reported transgene toxicity (see Methods in [13]). Second, their construct contains extensive *daf-16* intronic sequences that are not present in our construct. For example, intron 5 contains the β promoter element (this report). These sequences may affect the temporal and spatial patterns of gene expression. Third, Lin et al. expressed a GFP fusion protein. It may be that the GFP::DAF-16 fusion is functionally different from the native protein. We do not favor the model that overexpression of *daf-16α*::DAF16A1-4A causes constitutive dauer arrest. Control transgenes that were produced by the same injection concentration (2.5 ng/μl) but that expressed the wild-type DAF-16A1 proteins did not induce dauer arrest (Table 4). We have attempted to produce high levels of *daf-16* expression using a heat shock promoter construct. Transgenic animals carrying an integrated transgene of *hsp16-2*::DAF-16B arrested as partial or complete dauers only when subjected to repeat, periodic heat shock throughout their lives (unpublished data). This result argues that it is not easy to force expression of wild-type DAF-16 to a level sufficient to cause a dauer arrest constitutive phenotype.

The expression of mutant DAF-16 from the transgene suffers from some limitations. First, expression of *Ex[daf-16α::DAF16A1-4A]* is heavily selected against because of the arrest and lethality it causes. We could only maintain the stock by feeding them with *daf-16* RNAi bacteria. This RNAi treatment was not effective enough to completely shut down transgene expression (Table 4 and data not shown). Furthermore, there appears to be RNAi perdurance: the RNA interference of *daf-16* took more than one generation to wear off completely after transfer of animals from feeding RNAi bacteria (Table 4 and data not shown). Third, extrachromosomal transgenes tend to be lost during mitotic divisions, causing mosaicism and reducing overall expression [14]. This may explain why the *daf-16α*::DAF16A1-4A transgenic animals failed to precisely phenotype copy *daf-2(null)* mutant animals.

Mammalian insulin and insulin-like signaling regulate

Table 4

Dauer arrest phenotype caused by AKT phosphorylation incompetent DAF-16.

Transgene[a]	Reproductive development (N)	Dauer(-like) arrest (N)	L3/L2 (N)	L1/egg (N)
DAF-16(4A)[b]	1.1% (1)	26.8% (23)	68% (62)	14.6% (13)
No Transgene siblings[b]	94.9% (74)	0%	3.8% (3)	1.3% (1)
DAF-16(4A)[c]	44.2% (76)	19.2% (33)	36.6% (63)	n/c
DAF-16(WT)	94.0% (480)	0%	6% (33)	n/c

Animals were reared at 25°C and were scored 48.5–56 hr after egglay.

[a] All animals carried *daf-16(mgDf47)*. n/c means we had not particularly counted that class.

[b] Grandparents had been fed with *daf-16* dsRNA-expressing bacteria.

[c] Parents had been fed with *daf-16* dsRNA-expressing bacteria.

The DAF-16(WT) transgene was made by germline transformation with the PCR-generaged chimeric construct *daf-16α*::DAF-16A1 (2.6 μg/ml); DAF-16(4A) was made with *daf-16α*::DAF-16A1-4A (2.6 μg/ml) mutant fusion construct. The 4A mutant contains for S/T to A mutations that would abolish all potential sites of AKT phosphorylation [11]. *daf-16α*::DAF16A1-4A fusion transgenic animals were made by transforming GR1329 *daf-16(mgDf47)* animals with a mixture of 2.5 ng/μl *daf-16α*::DAF16A1-4A as a PCR product, 48 ng/μl pRF4, 34 ng/μl pTG96, and 11 ng/μl pPHgfp1 (expresses GFP in hypodermis [24]). Transgenic animals had to be maintained with *daf-16* RNAi *E. coli*. When grown on OP50 *E. coli*, transgenic animals are selected against by their dauer arrest phenotype. For feeding RNAi, a 970-bp PvuII/XhoI fragment common to all daf-16 isoforms was cloned into the SmaI site of plasmid pPD129.36, between T7 polymerase sites. The resulting plasmid, pT7-daf16common-T7, was used to transform *E. coli* strain HT115(DE3) and to make a bacterial strain, RX99. Feeding RNAi was performed on worm culture NG agar plates containing IPTG, ampicillin, and tetracyclin, as described by Timmons et al. [12].

DAF-16 homologous proteins by inhibiting nuclear import or activating nuclear export via AKT phosphorylation (reviewed in [1]). To see if this regulation is conserved in *C. elegans*, we assayed the subcellular localization of a functional GFP::DAF-16B fusion gene (driven by *daf-16α* promoter) in different *daf-2* genetic backgrounds. We found that, in *daf-16(mgDf47); daf-2(+)* animals, GFP::DAF-16B was predominantly cytoplasmic, with a high concentration around the nucleus (Figure 3A). In contrast, in a *daf-16(mgDf47); daf-2(e1370)* mutant background, where upstream insulin-like signaling is defective at 25°C, GFP::DAF-16B was concentrated in the nucleus (Figure 3B). This change in subcellular localization of GFP::DAF-16 appeared from the embryonic stage through late adulthood. Therefore, our results indicate that DAF-16 and its mammalian homologs are regulated by similar mechanisms. These results are in agreement with those reported by Lin et al. [13].

Our results indicate that phosphorylation of DAF-16 on all or some of the four S/T residues within AKT phosphorylation consensus sites is the major means by which insulin-like signaling transduces to *daf-16*. Furthermore, our results indicate that the absence of phosphorylation of these AKT consensus sites on DAF-16 is sufficient, independent of insulin signaling, to induce dauer arrest. This proves that *daf-16* is the major output of insulin signaling in *C. elegans* and suggests that FKHR, FKRHL1, and AFX may similarly be the major outputs of mammalian insulin signaling. Our results also argue that the AKT signaling output of DAF-2, and perhaps the orthologous mammalian insulin-like receptor tyrosine kinases, is the key output, rather than the wide range of other signaling pathways that have been suggested by biochemical analyses.

The *daf-7* TGF-β signaling pathway also regulates dauer arrest (reviewed in [15]). Although mutations in the TGF-β pathway can enhance the dauer arrest constitutive phenotype caused by reductions in *daf-2* insulin-like signaling, there is no evidence that demonstrates a mechanistic coupling between these two signaling pathways [3]. Furthermore, although both pathways regulate dauer arrest, only the insulin-like, but not the TGF-β, pathway affects life span (reviewed in [16]).

We checked if decreased TGF-β signaling also regulates DAF-16 nuclear localization. It did, although in a different manner from the insulin pathway. In a *daf-16(mgDf47); daf-7(m62)* background under dauer-inducing conditions, GFP::DAF-16B was almost exclusively localized in the nucleus throughout the animal but only during the L2d predauer stage Figure 3c. The L2d stage is an extension of the normal second larval stage that only forms under dauer-inducing environmental conditions. L2d animals, compared to normal L2, are slightly larger, due to prolonged feeding, and darker, apparently due to storage granules in the intestine. In the middle of the L2d stage, the animal becomes committed to enter the dauer stage rather than the L3 stage [17]. At all other stages in the *daf-7* mutant, including in the dauer stage, GFP::DAF-16 was largely excluded from the nucleus Figure 3d. This observation suggests that nuclear localization of DAF-16 is an important effector in the DAF-7 TGF-β signaling pathway as well. Indeed, although *daf-16; daf-7* double mutants are dauer arrest constitutive, the dauers that result are nevertheless incomplete. They lack pharyngeal constriction and form indistinct alae [8].

Our result indicates that the *daf-7* TGF-β signaling pathway crosstalks with the insulin pathway at a point upstream of DAF-16 nuclear localization. *daf-7* TGF-β signaling could act anywhere in the insulin signaling pathway, from the expression or secretion of any of the worm insulins to DAF-16 itself. For example, *daf-7* could cause a surge of *ins-1* expression in the L2d stage, which, as an antagonist, would shut down the *daf-2* pathway [18]. Alternatively, in cultured mammalian cells, TGF-β treatments lead to a rapid downregulation of PTEN transcript accumulation [19]. A similar mechanism could operate in the worm: loss of *daf-7* TGF-β signaling may increase *daf-18* PTEN expression at the L2d stage, causing a reduction in insulin signaling and AKT-(1,2) kinase activity, to affect DAF-16 nuclear accumulation. We stress that whatever the mechanism connecting *daf-7* to DAF-16 localization may be, it functions only at the L2d stage. This explains why mutations in the TGF-β pathway are synergistic with those in the insulin pathway only in the dauer formation, but not the longevity, phenotype.

Given the conservation that has been observed between worm and mammalian insulin signaling pathways, and given that mammalian GDF-8 (accession number NM010834) and GDF-11 (accession number AF028337) are candidate DAF-7 orthologs, it is possible that these TGF-β signals may also interact with insulin signaling in mammals.

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Exhibit B

DAF-16 recruits the CREB-binding protein coactivator complex to the insulin-like growth factor binding protein 1 promoter in HepG2 cells

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Insulin negatively regulates expression of the insulin-like growth factor binding protein 1 (IGFBP-1) gene by means of an insulin-responsive element (IRE) that also contributes to glucocorticoid stimulation of this gene. We find that the *Caenorhabditis elegans* protein DAF-16 binds the IGFBP-1·IRE with specificity similar to that of the forkhead (FKH) factor(s) that act both to enhance glucocorticoid responsiveness and to mediate the negative effect of insulin at this site. In HepG2 cells, DAF-16 and its mammalian homologs, FKHR, FKHRL1, and AFX, activate transcription through the IGFBP-1·IRE; this effect is inhibited by the viral oncoprotein E1A, but not by mutants of E1A that fail to interact with the coactivator p300/CREB-binding protein (CBP). We show that DAF-16 and FKHR can interact with both the KIX and E1A/SRC interaction domains of p300/CBP, as well as the steroid receptor coactivator (SRC). A C-terminal deletion mutant of DAF-16 that is nonfunctional in *C. elegans* fails to bind the KIX domain of CBP, fails to activate transcription through the IGFBP-1·IRE, and inhibits activation of the IGFBP-1 promoter by glucocorticoids. Thus, the interaction of DAF-16 homologs with the KIX domain of CBP is essential to basal and glucocorticoid-stimulated transactivation. Although AFX interacts with the KIX domain of CBP, it does not interact with SRC and does not respond to glucocorticoids or insulin. Thus, we conclude that DAF-16 and FKHR act as accessory factors to the glucocorticoid response, by recruiting the p300/CBP/SRC coactivator complex to an FKH factor site in the IGFBP-1 promoter, which allows the cell to integrate the effects of glucocorticoids and insulin on genes that carry this site.

Insulin signaling via the phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (PKB) pathway has diverse effects on cellular metabolism and apoptosis (1, 2). A major role of insulin is to act in opposition to the catabolic effects of cAMP and glucocorticoids, agents that stimulate liver gluconeogenesis. The rate-limiting step in gluconeogenesis is catalyzed by the phospho*enol*pyruvate carboxykinase (GTP) (PEPCK; EC 4.1.1.32) gene. The insulin-like growth factor binding protein 1 (IGFBP-1) gene indirectly promotes gluconeogenesis by binding insulin-like growth factor (IGF)-I and -II and inhibiting their insulin-like effects. Expression of the PEPCK and IGFBP-1 genes is controlled at the transcriptional level by a complex regulatory mechanism in which glucocorticoids activate and insulin inhibits gene expression (3–6).

In the case of the PEPCK gene, the response to both glucocorticoids and insulin is mediated by the accessory factor II (AFII) site, located upstream of the glucocorticoid-response element (GRE); this site is also referred to as the PEPCK insulin-response sequence, IRS-1 (7). Similarly the response of the IGFBP-1 promoter to glucocorticoids and insulin is mediated by one site, the insulin-response element (IRE) site located upstream of its GRE (5, 8). Biochemical evidence first showed that the forkhead (FKH) hepatic nuclear factor (HNF)3β binds the IRE in the PEPCK and IGFBP-1 genes and enhances the effect of glucocorticoids on gene transcription (9, 10). Thus, early efforts to identify the mediator of glucocorticoid and insulin action at this site focused on HNF3β. The effect of HNF3β to enhance the action of glucocorticoids can be mimicked by GAL4-HNF3β, if a GAL4 DNA-binding site is substituted for the AFII site in the PEPCK gene. This observation provides strong support for a role of HNF3β as an accessory factor for the glucocorticoid response (11). However, if a single protein mediates the response to both glucocorticoids and insulin at this site, it is unlikely to be HNF3β, inasmuch as certain HNF3 sites fail to mediate the negative effect of insulin and other HNF3 sites actually confer insulin inducibility to a reporter gene (12, 13).

Genetic evidence identifying the transcriptional outputs of insulin-like factors in *Caenorhabditis elegans* indicates that the FKH transcription factor DAF-16 is the major target downstream of the *C. elegans daf-2* (insulin receptor), *age-1* (PI 3-kinase), PKB/Akt (AKT1/AKT2)-dependent pathway (14, 15). The effect of mutations in *daf-2* and *age-1* is reversed by loss of function mutations in *C. elegans daf*-16. Thus, insulin signaling via this pathway negatively regulates the activity of DAF-16 (14–17). Several groups simultaneously showed that close relatives of DAF-16, including FKHRL1 (18), AFX (19), and FKHR (13, 20–23) are direct targets of insulin/insulin-like growth factor signaling to PI 3-kinase and PKB. These factors can activate transcription via the IGFBP-1·IRE. Phosphorylation of FKHRL1 (18), FKHR/FKHR1 (23), and AFX (24) by insulin/insulin-like growth factor signaling or overexpression of PKB promotes export of these proteins from the nucleus, thereby preventing their transcriptional effect. Therefore DAF-16-like factors are likely candidates for the protein that integrates the response to glucocorticoids and insulin at the IGFBP-1·IRE.

While the three mammalian homologs of DAF-16 show up to 60% homology to DAF-16 within the FKH DNA-binding domain and marked conservation of their AKT/PKB phosphorylation sites, these homologs are not conserved compared with DAF-16 outside of these regions (14, 15). Thus there is uncertainty as to which of the mammalian FKH proteins is functionally most similar to DAF-16. Therefore, we compared the hormone response of DAF-16 and its mammalian homologs in HepG2

Abbreviations: CBP, CREB-binding protein; SRC, steroid receptor coactivator; GRE, glucocorticoid-response element; IRE, insulin-response element; IGFBP-1, insulin-like growth factor binding protein 1; PEPCK, phospho*enol*pyruvate carboxykinase; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; GST, glutathione *S*-transferase; FKH, forkhead; HNF, hepatic nuclear factor; CMV, cytomegalovirus.

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cells, hoping that DAF-16 would behave as an ortholog of the mammalian DAF-16-like factors and that we could proceed to examine the effect of nonfunctional DAF-16 homologs on hormone-regulated IGFBP-1 gene expression.

We find that DAF-16 and FKHR are most similar in their ability to activate gene transcription and modulate the response of the IGFBP-1 promoter to glucocorticoids and insulin. Both DAF-16 and FKHR bind to the KIX domain of CREB-binding protein (CBP) and to steroid receptor coactivator (SRC)-1. A C-terminal deletion mutant of DAF-16 (15), which fails to interact with the KIX domain of CBP, is transcriptionally inactive in mammalian cells and prevents the effect of glucocorticoids to stimulate IGFBP-1 gene expression. Furthermore, we find that AFX, a homolog of DAF-16 that fails to bind SRC-1, also fails to respond to glucocorticoids and insulin. Thus we have uncovered a link between DAF-16 homologs and their ability to recruit the p300/CBP/SRC coactivator complex that could explain their ability to integrate complex hormonal signals.

Materials and Methods

Constructs. The DAF-16a1 *Hin*dIII/*Nhe*I insert from pGEM-FLAG-DAF-16a1 was ligated into the *Hin*dIII/*Xba*I site of pcDNA3.1(+) (Invitrogen) to generate pcDNA3.1+FLAG-DAF-16a1. The DAF-16a1 *Bst*YI insert from pGEM-FLAG-DAF-16a1 was ligated into the *Bam*HI site of pGEX-4T-1 (Pharmacia) to generate pGEX4T-DAF-16a1. Phosphorylation site mutants were prepared by using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Primers were T54A (so164/165): GAT CGG TGC AAT GCT TGG CCA ATG CGT/ACG CAT TGG CCA AGC ATT GCA CCG ATC; S240A/S242A (so200/201): CGT ACA CGT GAA CGA GCC AAT GCT ATT GAG ACG ACT AC/GTA GTC GTC TCA ATA GCA TTG GCT CGT TCA CGT GTA CG; and S314A (so168/169): CCC CGA ACT CAA GCT AAC CTC TCG ATT/AAT CGA GAG GTT AGC TTG AGT TCG GGG.

Sources of plasmids: The rat IGFBP-1 promoter (nucleotides −925 to +79) cloned in PGL3-LUC was a gift from Matthew Rechler, National Institutes of Health, Bethesda, MD). FKHR, FKHRL1, and AFX were obtained from K. Arden (Univ. of California, San Diego). pCMV·HNF3α was a gift from J. Darnell (Rockefeller University, New York). Constitutively active pCMV6·gag-PKB was a gift from J. R. Woodgett (British Columbia Cancer Agency, Jack Bell Research Centre, Vancouver, Canada). CMV·HA-tagged p300 was a gift from Marc Montminy (Joslin Diabetes Center, Boston). Plasmids encoding glutathione *S*-transferase (GST)-SRC (25) and GST-CBP constructs containing the C/H1 domain (amino acids 312–450), the KIX domain (amino acids 450–684), and the C/H3 domain (amino acids 1890–2441) were gifts from Tony Hollenberg and Fred Wondisford (Beth Israel Hospital, Boston) (26). The pcDNA·SRC-1 (27) plasmid was a gift from William Chin (Brigham and Women's Hospital, Boston). CMV·E1A and CMV·E1A Δ2–36 (28), pCMX·VP-16 and pCMX·VP-16 p300, which are driven by a cytomegalovirus (CMV) promoter, were gifts from D. Livingston (Dana–Farber Cancer Institute, Boston).

Tissue Culture, Transfection, and Reporter Assays. Hepatoma (HepG2) cells were purchased from the American Type Culture Collection. HepG2 cells were cultured in minimal essential medium (MEM) supplemented with nonessential amino acids, glutamine, sodium pyruvate, penicillin/streptomycin, and 10% fetal bovine serum (FBS).

HepG2 cells (passages 2–6) were seeded on 30-mm six-well plates at 50% confluence. Twenty-four hours later, the cells were incubated with Dulbecco's modified Eagle's medium (DMEM) supplemented with charcoal-treated 10% FBS. Two hours later, the cells were exposed to a DNA/calcium phosphate precipitate for 4 h and then shocked with 20% (vol/vol) dimethyl sulfoxide (DMSO) in PBS for 1 min. HepG2 cells were cotransfected with 10 μg/ml IGFBP-1·IRE or the IGFBP-1·luciferase plasmid, and 1 μg/ml pcDNA expression vector alone, or vectors encoding DAF-16, FKHR, mFKHRL1, AFX, or pCMV·HNF3α per ml of precipitate. RSV·GH or RSV-β-galactosidase (RSV, Rous sarcoma virus; GH, growth hormone) was included as a cotransfected control. In some experiments a CMV expression vector alone (0.2 μg/ml) or expression vector encoding E1A wild type, E1A Δ2–36, or active pcDNA-PKB was included. The cells were washed twice with PBS before the addition of serum-free DMEM supplemented with 0.1% crystalline BSA. Luciferase gene activity was measured 24 h after the transfection by using a luciferase assay kit (Promega). Each transfection was performed in triplicate and repeated at least three times. Cells were harvested 22 h after the addition of insulin (1 milliunit/ml) or dexamethasone (0.5 μM). The GH RIA and β-galactosidase assays (29) were performed as previously described. HEK293 cells were transfected by using the modification of the calcium phosphate precipitation protocol described above except that the cells were not shocked with 20% DMSO.

Protein Interaction Assay. The pcDNA·DAF-16, pCMV·p300, and pcDNA·SRC1 plasmids were used for *in vitro* synthesis of proteins in rabbit reticulocyte lysate by using protocols described by the supplier (Promega). GST-fusion proteins were prepared as described previously (29). The quality of each preparation was examined by SDS/PAGE, and the GST proteins were matched for molar content in the crude preparation. GST pull-down assays were performed by using a method described by Lai and Herr (30) with modifications described previously (29). The amount of GST-fusion protein absorbed to the beads was quantitated by subjecting a fraction of the proteins released to SDS/PAGE followed by staining with Coomassie blue.

Yeast Two-Hybrid Screen. In the yeast two-hybrid screen, a fusion between GAL4 DNA-binding domain and amino acid 397–683 of *C. elegans* CBP-1 was constructed by using the PAS2-1 vector (CLONTECH) and used as bait to screen a mixed-stage *C. elegans* library (kindly provided by Robert Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK). The library was screened by using the reagents and protocols provided in the Matchmaker Two-Hybrid System 2 kit (CLONTECH).

Results

DAF-16 Homologs Modulate the Effect of Glucocorticoids and Insulin on the IGFBP-1 Gene. As previously shown for its mammalian homolog FKHR (31), DAF-16 binds the wild-type IGFBP-1·IRE and not a mutant that eliminates the effect of glucocorticoids and insulin on this gene (data not shown). To determine which of three mammalian homologs of DAF-16—FKHR, FKHRL1, or AFX—was most similar in function to DAF-16, we compared their effects on glucocorticoid- and insulin-responsive gene transcription. In three independent experiments, DAF-16 stimulated IGFBP-1 promoter activity by 8- to 10-fold, and insulin inhibited this effect by 90% (Fig. 1*A*, compare bar A to bars C and D). The abilities of DAF-16 and FKHR to activate IGFBP-1 gene expression were identical in magnitude (Fig. 1*A*, bars C and E), as were the effects of insulin to inhibit DAF-16 and FKHR by 90% (Fig. 1*A*, bars D and F). The effect of insulin on FKHRL1 was consistently less pronounced than its effect on FKHR (Fig. 1*A*, bars G, H and E, F). In contrast, insulin did not inhibit activation of the IGFBP-1 promoter by AFX at all (bars I and J). Thus, in HepG2 cells, FKHR is the mammalian homolog that functions most like its *C. elegans* homolog, DAF-16.

The effect of DAF-16 and its homologs on glucocorticoid-responsive gene transcription was assessed by using a concen-

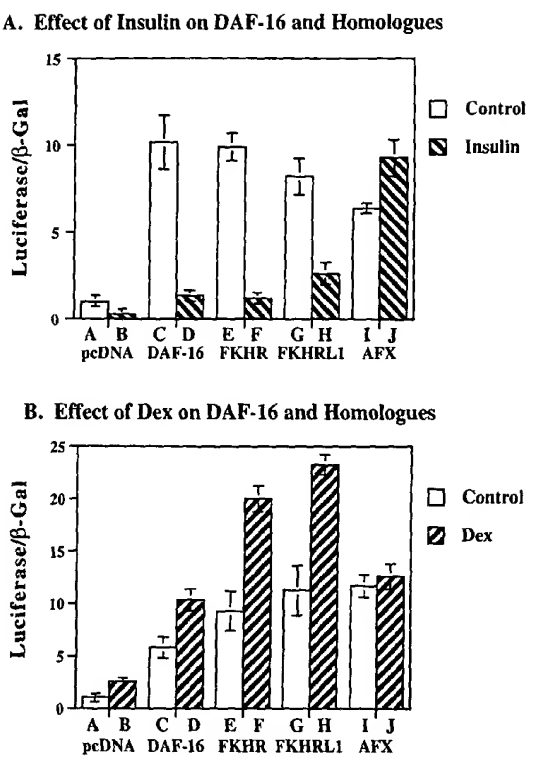

Fig. 1. Effect of DAF-16 homologs on insulin- and glucocorticoid-responsive gene transcription. HepG2 were cotransfected with a construct encoding the native IGFBP-1 promoter (10 μg/ml) driving luciferase gene expression and the pcDNA expression vector alone (1 μg/ml), or the expression vectors pcDNA·DAF-16, pcDNA·FKHR, pcDNA·FKHRL1, and pcDNA·AFX (1 μg/ml). In *A*, insulin (1 milliunit/ml) was added to serum-starved cells during the last 18 h of incubation. In *B*, cells were exposed to dexamethasone (0.5 μM) for 18 h. The effect of these agents on endogenous proteins, pcDNA (bars A and B), or the exogenous proteins encoded by pcDNA·DAF-16 (bars C and D) and its three mammalian homologs, pcDNA·FKHRL1 (bars E and F), pcDNA·FKHR (bars G and H), and pcDNA·AFX (bars I and J) is shown. Luciferase activity was corrected for β-galactosidase gene expression. The data shown are representative of three experiments.

tration of each designed to achieve less than maximal stimulation of basal activity. Again DAF-16 homologs stimulated basal gene expression by 6- to 10-fold. Under these conditions, dexamethasone enhanced the effect of DAF-16 by 70%, and the effect of FKHR and FKHRL1 by 100% (Fig. 1*B*, bars D, F, and H, respectively). Thus we conclude that in the absence of insulin, FKHR and FKHRL1 facilitate glucocorticoid activation of the IGFBP-1 promoter. In contrast, although AFX stimulated basal transcription of the IGFBP-1 promoter by 10-fold, this homolog failed to show glucocorticoid-stimulated gene expression under any conditions. Thus we conclude that individual DAF-16 homologs mediate distinct regulatory functions.

E1A Interacts with p300/CBP and Blocks Activation of IGFBP-1 Gene Transcription by DAF-16. Mutations of the IGFBP-1·IRE impair glucocorticoid-stimulated gene expression, pointing to an enhancing effect of the protein complex bound at the IRE site on glucocorticoid-induced transcription. To determine whether binding of DAF-16-like proteins to the IRE modulates the effect of glucocorticoids, we overexpressed wild-type and mutant DAF-16 in HepG2 cells. In the absence of exogenous factors, IGFBP-1 promoter activity was stimulated 3-fold by glucocorticoids (Fig. 2*A*, bars A and B). Wild-type DAF-16 increased

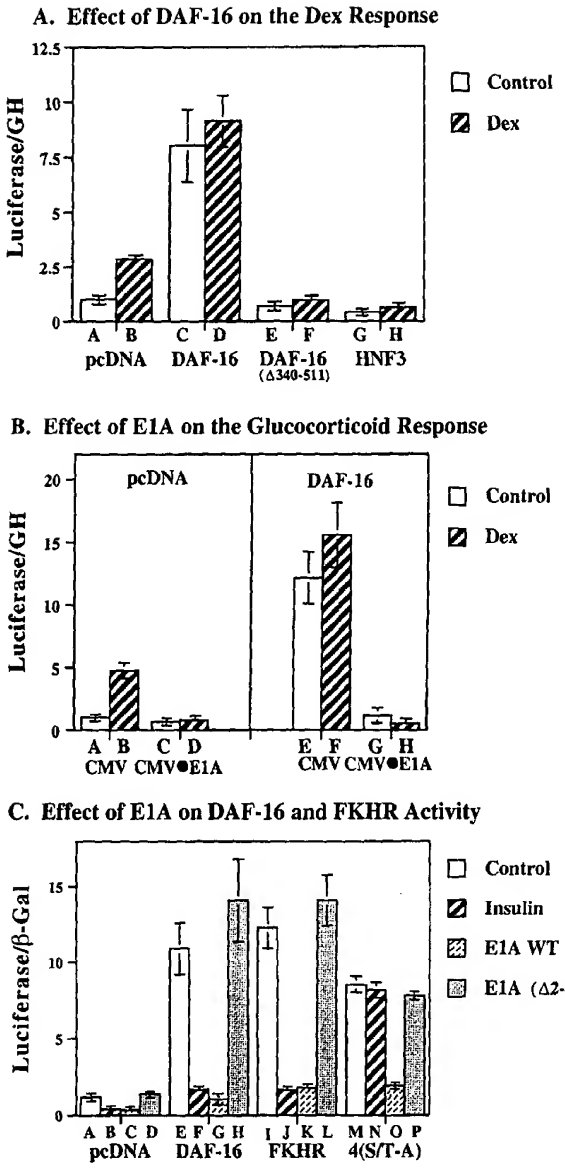

Fig. 2. (*A*) Effect of DAF-16 and HNF3α on insulin-responsive gene transcription. HepG2 cells were incubated in the presence or absence of insulin (1 milliunit/ml) for 16 h before harvesting. Luciferase activity recovered in the presence of pcDNA (bars A and B), pcDNA·DAF-16 (bars C and D), pcDNA·DAF-16 (Δ340–511) (bars E and F), and HNF3α (bars G and H) is shown. (*B*) Effect of E1A on dexamethasone-responsive gene transcription. Cells were transfected with the native IGFBP-1 promoter driving luciferase gene expression (15 μg/ml), and expression vectors including pcDNA alone (bars A–D) or pcDNA·DAF-16 (bars E–H); and CMV alone (1 μg/ml) (bars A, B, E, and F); or CMV·E1A (bars C, D, G, and H). Cells were inoculated with (bars B, D, F, and H) and without (bars A, C, E, and G) dexamethasone (0.5 μM) for 18 h. Luciferase activity is shown corrected for growth hormone (GH) and normalized to the control value for pcDNA alone. (*C*) DAF-16 gene expression is inhibited by insulin and by wild-type E1A but not by E1A Δ2–36. HepG2 cells were transiently cotransfected with the native IGFBP-1 promoter-luciferase gene (10 μg/ml), and the pcDNA expression vector alone (1 μg/ml) (bars A–D), or wild-type pcDNA·DAF-16 (bars E–H), or pcDNA·FKHR (bars I–L) or pcDNA·DAF-16 4(S/T-A) (bars M–P). Control and insulin-stimulated activity was assessed in the presence of the expression vector CMV alone (0.2 μg/ml; bars A, B, E, F, I, J, M, and N). The effect of wild-type CMV·E1A (bars C, G, K, and O) or a derivative of E1A that fails to interact with CBP, CMV·E1A Δ2–36 (bars D, H, L, and P) is shown.

Table 1. CBP interacts with DAF-16 in yeast and mammalian two-hybrid system

Yeast two-hybrid system				Mammalian two-hybrid system			
					Activity (Luc/β-gal)		
Clone	Amino acids	No. of clones	*lacZ*⁺/*HIS*	GAL4·DAF-16 derivative	VP16	VP16·p300	-Fold effect of p300
DAF-16	176–508	1	++/+	GAL4·BD	0.9 ± 0.09	0.91 ± 0.03	1
F38A6.3 (HIF-1α)	104–343	5	++/+	GAL4·DAF-16 wild type	31 ± 1.89	284 ± 5.03	9
T01B10.4 (HNF4)	51–450	2	+/+	GAL4·DAF-16 4(S/T-A)	57 ± 2.23	328 ± 22.4	6
ZK1290.4 (NF-1)	525–1026	1	++/+	GAL4·DAF-16 (Δ340–511)	4.5 ± 0.14	12.5 ± 0.93	3

Yeast two-hybrid system: Two million independent colonies were screened and 92 positive clones were isolated by using the reporter genes *lacZ* and *HIS3*. Yeast plasmids encoding the 92 "preys" were rescued into *Escherichia coli* HB101 and used in new yeast transformation experiments to confirm the two-hybrid interaction. On the second round of screening 46 clones were positive with GAL4·CBP-1, but not with the GAL4 DNA-binding domain alone or GAL4LAM5'-1 (CLONTECH). Twenty-two of the 46 clones encoded putative *C. elegans* transcription factors, some of which have known mammalian homologs. A partial list is shown in this table. The predicted open reading frames (ORFs) of clones F38A6.3, T01B10.4, and ZK1290.4 encode proteins related to mammalian HIF-1α, HNF4, and NF-1, respectively. The length of the ORFs of the isolated clones, the number of independent clones identified for each interacting molecule, and the relative strength of the interactions is shown in columns 2, 3, and 4, respectively. Mammalian two-hybrid system: GAL4·DAF-16 derivatives (2 μg) were cotransfected into HEK 293 cells with VP-16 alone or VP-16·p300 (2.5 μg) and the luciferase (15 μg) or β-galactosidase (2.5 μg) reporter genes as described in the text. Cells were assayed for luciferase (Luc) and β-galactosidase (β-gal) activity. Luciferase activity was corrected for coexpression of β-galactosidase activity (±SEM). The fold effect indicates the specific interaction of the GAL4·DAF-16 derivative with VP-16·p300 compared to VP-16 alone.

basal promoter activity by 8-fold, and there was no additional effect of glucocorticoids (bars C and D). Mutant DAF-16 (Δ340–511) had no effect on basal activity, and it prevented the effect of glucocorticoids to stimulate IGFBP-1 gene expression by means of endogenous factors (compare bars E, F to A, B). Thus the C-terminal domain of DAF-16 appears to be required for basal- and glucocorticoid-responsive activation of the IGFBP-1 promoter. The effect of HNF3α, a FKH family DNA-binding protein (bars G and H) was similar to that of DAF-16 (Δ340–511) in that occupation of the site by HNF3α also prevented the effect of endogenous factors.

We speculated that the proposed ability of FKH proteins to act as accessory factors for the stimulatory effect of glucocorticoids by means of the IGFBP-1·IRE might result from recruitment of the p300/CBP coactivator complex to this site, as is seen with the glucocorticoid receptor (32, 33). If so, one interpretation of the observation that glucocorticoids do not further stimulate the IGFBP-1 gene in the presence of DAF-16 (compare bars A, B and C, D in Fig. 2*A*) would be that both DAF-16 and glucocorticoids act by the same mechanism. Therefore, we examined whether E1A, a viral oncoprotein that interacts with and sequesters p300/CBP, could inhibit the stimulatory effect of DAF-16 or glucocorticoids on IGFBP-1 gene expression. In the absence of DAF-16, basal IGFBP-1 promoter activity was stimulated 4-fold by glucocorticoids (Fig. 2*B*, bars A and B), and this effect was inhibited by wild-type E1A (Fig. 2*B*, compare bars A and B to C and D). In the presence of DAF-16, IGFBP-1 gene expression was stimulated 8-fold, and no further activation was observed in the presence of glucocorticoids (compare bars A and B to E and F in Fig. 2*B*). E1A inhibited the effect of DAF-16, independent of the addition of glucocorticoids (Fig. 2*B*, compare bars E and F to bars G and H).

Next we compared the effect of wild-type E1A and a derivative of E1A missing the N-terminal p300/CBP-interaction domain, E1A Δ2–36 (28, 34), on IGFBP-1 promoter activity. In the absence of DAF-16, wild-type E1A inhibited the effect of endogenous factors on IGFBP-1 promoter activity by 80% (Fig. 2*C*, bars A and C), whereas no inhibition was seen by E1A Δ2–36 (Fig. 2*C*, bars A and D). Thus, in HepG2 cells, basal activation of the IGFBP-1 promoter by endogenous factors is likely to depend on p300/CBP. DAF-16 activated expression of the IGFBP-1 promoter 8-fold (Fig. 2*C*, compare A to E), and its homolog FKHR activated expression 10-fold (Fig. 2*C*, compare A to I). The effect of DAF-16 and FKHR on IGFBP-1 promoter activity was inhibited 90% by wild-type E1A (Fig. 2*C*, compare bars E to G and I to K). Again, E1A Δ2–36 had no effect on DAF-16- or FKHR-stimulated IGFBP-1 promoter activity (Fig. 2*C*, compare bars E to H and I to L). A similar pattern of inhibition by wild-type E1A, but not mutant E1A Δ2–36 was observed with all of the mammalian homologs of DAF-16 (data not shown). This observation suggested that, in HepG2 cells, binding of the p300/CBP coactivator complex to DAF-16 and FKHR is essential for the ability of DAF-16 to activate IGFBP-1 gene transcription.

We compared the effect of insulin and E1A on the activity of wild-type DAF-16 and an insulin-insensitive mutant DAF-16 4(S/T-A), which carries an alanine substitution within its four consensus AKT/PKB phosphorylation sites (Thr-54, Ser-240, Thr-242, and Ser-314). While wild-type DAF-16 was inhibited 85% by insulin (Fig. 2*C*, bars E and F), the activity of the DAF-16 4(S/T-A) site mutant was not affected by insulin (bars M and N). This observation confirms previous reports that the inhibitory effect of insulin on the wild-type DAF-16 protein is dependent on phosphorylation of DAF-16 at one or more of its putative AKT/PKB sites in HepG2 cells (13). Although insulin had no effect on the activity of DAF-16 4(S/T-A), wild-type E1A, but not mutant E1A Δ2–36, inhibited the activity of this mutant by 70% (Fig. 2*C*, compare bar M to bar O and P). Thus, in HepG2 cells, phosphorylation of the AKT sites on DAF-16 is not required for the interaction of p300/CBP with DAF-16 and activation of the IGFBP-1 promoter or for E1A inhibition of this effect. In contrast, insulin inhibits DAF-16 activity by a mechanism that requires the AKT sites in DAF-16.

p300/CBP Interacts with DAF-16 *in Vitro* and *in Vivo*. In an independent series of experiments aimed at identifying important CBP interactors in *C. elegans* we obtained further evidence that CBP and DAF-16 interact in cellular systems. Using the N-terminal region of *C. elegans* CBP-1 (amino acids 397–683), which contains the C/H1 and KIX domains as bait, we recovered 22 *C. elegans* transcription factors that interact with CBP-1, 4 of which were related to mammalian factors (Table 1). In addition to DAF-16, *C. elegans* clones F38A6.3, TO1B10.4, and 2K1290.4 were recovered from the screen and found to bear striking homology to hepatocyte nuclear factor 4 (HNF4), hypoxia-inducible factor-1α (HIF-1α), and nuclear factor 1 (NF-1), respectively. The KIX domain of mammalian CBP has previously been shown to interact with HNF4 and HIF-1α in mammalian systems (35, 36). Thus, we conclude that the interaction of DAF-16 with CBP-1 is comparable to that for other known CBP-interacting proteins (Table 1).

We used the mammalian two-hybrid system to confirm that CBP interacts with DAF-16 in mammalian cells. DAF-16 was expressed

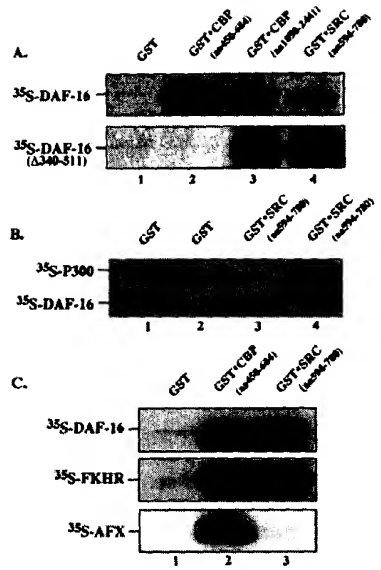

Fig. 3. DAF-16 interacts with p300 and SRC. (*A*) Interaction of [^{35}S]methionine-labeled DAF-16 with the KIX and C/H3 interaction domains of GST·CBP. *In vitro* translated [^{35}S]methionine-labeled DAF-16 or DAF-16 (Δ340–511) was incubated with GST (lane 1); or GST·CBP (KIX), which encodes amino acids 450–684 of CBP (lane 2); or GST·CBP (C/H3), which encodes amino acids 1890–2441 of CBP (lane 3); or GST·SRC, which encodes amino acids 594–780 (lane 4). The bound proteins were washed, eluted, and subjected to SDS/PAGE as described in the text. The autoradiograph of a dried gel is shown. (*B*) Interaction of GST·SRC with [^{35}S]methionine-labeled p300 and DAF-16. *In vitro* translated [^{35}S]methionine-labeled p300 (lanes 2 and 4) or DAF-16 (lanes 1 and 3) was incubated with bacterially produced GST (lanes 1 and 2) or GST·SRC (amino acids 594–780) (lanes 3 and 4) bound to glutathione-Sepharose beads. Eluted proteins are shown. (*C*) Interaction of [^{35}S]methionine-labeled DAF-16, FKHR, and AFX with GST·CBP (KIX) and GST·SRC. [^{35}S]Methionine-labeled DAF-16, FKHR, and AFX were incubated with GST (lane 1), GST·KIX (lane 2), or GST·SRC (lane 3) bound to glutathione-Sepharose beads. Eluted proteins are shown.

as a fusion protein with the GAL4 DNA-binding domain and full-length CBP as a fusion protein with the VP-16 activation domain. GAL4·DAF-16 derivatives and the VP-16 derivatives were cotransfected into HEK293 cells in the presence of a luciferase reporter gene driven by a GAL4 DNA-binding site. There was no effect of the VP-16·CBP fusion gene on the GAL4 DNA-binding domain construct alone. In the presence of GAL4·DAF-16 the CBP·VP16 fusion gene activated gene transcription by 9-fold compared with VP-16 alone. Mutation of the AKT sites in DAF-16 had little effect on the ability of CBP·VP16 to activate gene transcription; activation by CBP·VP16 was 6-fold over that of VP-16 alone. In contrast, the ability of the C-terminal DAF-16 mutant to interact with CBP·VP16 was markedly decreased compared with wild-type DAF-16. The fact that the interaction of CBP with mutant DAF-16 was not abolished can be explained by the fact that both the KIX domain and the E1A/SRC domain of p300 interact with DAF-16; thus, the C-terminal mutant can still interact with the E1A/SRC domain of p300 (see Fig. 3).

To determine whether CBP could interact directly with DAF-16 and to map the domain involved *in vitro*, the interaction of CBP with DAF-16 was confirmed by using the GST pull-down assay. Crude GST fusion proteins that include three major interaction domains of CBP·C/H1 (amino acids 312–450), GST-CBP·KIX (amino acids 450–684), and GST-CBP·C/H3 (amino acids 1890–2441) (25), respectively, were bound to glutathione-Sepharose columns and incubated with [^{35}S]methionine-labeled DAF-16. DAF-16 did not bind GST alone (Fig. 3*A*, lane 1), nor did it bind the C/H1 domain of CBP (data not shown). As expected, however, DAF-16 interacts with the KIX domain of CBP (Fig. 3*A*, lane 2); approximately 10% of the applied proteins were recovered (data not shown). DAF-16 also interacts with the C/H3 domain of GST·CBP (Fig. 3*A*, lane 3), the domain that interacts with E1A and SRC. Thus, there are two domains within CBP that interact with DAF-16.

In mammalian cells p300/CBP is known to interact with SRC and certain acetyltransferases to form a coactivator complex that is essential for activation of gene transcription by members of the nuclear receptor superfamily (37, 38). To determine whether recruitment of p300/CBP by DAF-16 might be reinforced by an indirect interaction with SRC, we incubated [^{35}S]methionine-labeled DAF-16 with GST·SRC and found that DAF-16 could indeed interact with SRC (Fig. 3*A*, lane 4). When we compared the ability of p300/CBP and DAF-16 to bind GST·SRC, we found that binding of *in vitro* translated DAF-16 to GST·SRC (Fig. 3*B*, lane 3) was comparable to binding of p300 with GST·SRC (Fig. 3*B*, lane 4). Thus, we conclude that DAF-16 interacts with p300/CBP and SRC.

In contrast to wild-type DAF-16, the transcriptionally inactive DAF-16 (Δ340–511) failed to bind the KIX domain of CBP (amino acids 450–684) (Fig. 3*A*, lane 2), but it did bind the E1A/SRC interaction domain of GST·CBP (amino acids 1890–2441) (lane 3) and GST·SRC (lanes 4). Thus wild-type DAF-16 interacts with two distinct sites on CBP, whereas the inactive C-terminal truncation mutant of DAF-16 interacts with the E1A/SRC domain of CBP, but not the KIX domain. Therefore, we conclude that basal and glucocorticoid-mediated activation of the IGFBP-1 promoter requires an interaction of DAF-16 with the KIX domain of DAF-16.

Binding of *in vitro* translated DAF-16, FKHR, and AFX to the KIX domain of CBP and the interaction domain of SRC is compared in Fig. 3*C*. DAF-16 and FKHR bind to both the KIX domain of CBP and the interaction domain of SRC (Fig. 3*C*, lanes 2 and 3), whereas AFX binds only CBP·KIX, and not SRC (lanes 2 and 3). We conclude that the interaction of AFX with the KIX domain of CBP is sufficient for basal transcriptional activity but not for hormone-regulated activity.

Discussion

We show that in HepG2 cells, DAF-16, a member of the FKH family of transcriptional regulators, can recruit the p300/CBP coactivator complex to the IGFBP-1·IRE, a site that both enhances the positive effect of glucocorticoids and mediates the negative effect of insulin on the IGFBP-1 gene. Consistent with previously published findings that insulin inhibits the activity of FKHR, a mammalian homolog of DAF-16 (13), we show that the inhibitory effects of insulin on DAF-16 and FKHR were identical, ranging from 70% to 90%. However, in contrast to previously published findings where insulin inhibited the activity of AFX by 40% in NIH 3T3 cells (19), insulin did not inhibit activation of the IGFBP-1 gene by AFX in HepG2 cells. Thus we conclude that the mammalian homologs of DAF-16 are not functionally equivalent.

In the absence of insulin, we find that DAF-16-like proteins recruit the p300/CBP coactivator complex to the IGFBP-1 gene. Recruitment of the p300/CBP coactivator complex is essential for cAMP- and glucocorticoid-responsive gene transcription (32, 39). In the presence of dexamethasone, the glucocorticoid receptor can interact with the coactivators SRC/GRIP/CBP and their associated histone acetyltransferases (40, 41) to its target genes. Thus our observation that DAF-16 can recruit the coactivator complex to the site that enhances glucocorticoid responsiveness implies that one function of DAF-16-like proteins is to provide additional binding sites for the coactivator complex on the IGFBP-1 promoter. Two findings support this view. First, the nonfunctional mutant of DAF-16 lacking the CBP–KIX interaction domain acts as a dominant inhibitor of the glucocorticoid response. Second, in the presence of saturating amounts of wild-type DAF-16, glucocorticoids have no further stimulatory

effect on IGFBP-1 gene expression, suggesting that the glucocorticoid receptor and DAF-16 act by a common mechanism. Accordingly, at subsaturating concentrations of DAF-16 and FKHR, we were able to demonstrate an enhancing effect of these factors on glucocorticoid-responsive gene transcription.

Although AFX interacts with the KIX domain of CBP, it does not respond to glucocorticoids or insulin. Thus the interaction with the KIX domain of CBP appears to be necessary, but not sufficient, for glucocorticoid regulation of IGFBP-1 gene transcription. We conclude that the interaction of DAF-16-like proteins with the KIX domain of CBP is essential for basal activation of IGFBP-1 gene transcription in HepG2 cells, but it is not sufficient for hormone-regulated gene expression. Examination of the DAF-16 amino acid sequence shows several regions that carry variations on the interaction motif found in the accessory factor II domain (42) of nuclear receptors that interact with SRC (33, 43). SRC can, in turn, interact with the C/H3 domain of p300/CBP (37); thus CBP is indirectly recruited to nuclear receptors by SRC. We find that DAF-16 and FKHR can bind directly to SRC as well as CBP. The ability of DAF-16-like factors to mediate the effect of both glucocorticoids and insulin on the IGFBP-1 gene correlates with this interaction as opposed to the interaction of DAF-16-like factors with the KIX domain of CBP. Specifically, the DAF-16 homolog AFX fails to bind SRC *in vitro* and fails to mediate the effect of glucocorticoids and insulin on IGFBP-1 gene transcription. Thus, distinct DAF-16 family members appear to play distinct roles on their target genes.

The physiologic relevance of the interaction of DAF-16 with SRC and CBP as it pertains to hormone-regulated gene expression is not yet known. However, a precedent for the suggestion that recruitment of CBP is essential for hormone-regulated stimulation of PIT-1 gene transcription by agents such as cAMP and insulin, which usually show opposing effects on gene transcription, has been established in GH4 cells (44). For example, the recruitment of CBP is essential for positive regulation of PIT-1 by cAMP, whereas recruitment of CBP by SRC is critical for positive regulation of PIT-1 by insulin. Thus, it seems reasonable to suggest that recruitment of distinct coactivator or corepressor complexes may play a role in mediating multihormonal regulation of IGFBP-1 gene transcription.

The PEPCK gene carries two weak GREs and three well-described accessory factor binding sites that are essential for the effect of glucocorticoids to activate gene expression (7, 11). The accessory factor I (AFI) site binds HNF4 and the accessory factor II (AFII) site binds FKH domain factors such as HNF3 (11) and FKHR (31). In mammalian cells, HNF4 interacts with the CH/1 domain of CBP and with SRC/GRIP (45). Using the yeast two-hybrid system to find targets of CBP in *C. elegans*, we found that the N terminus of CBP selected a clone that encodes a protein related to HNF4 as well as DAF-16. Our observation that DAF-16 and FKHR can interact with CBP and SRC just as the nuclear receptor HNF4 can suggests a common mechanism whereby either family of factors could enhance glucocorticoid-responsive gene expression by recruiting the CBP coactivator complex to promoters with relatively weak GREs.

We propose then that in HepG2 cells, DAF-16 and its mammalian homologs activate basal transcription of the IGFBP-1 gene by recruiting p300/CBP to the promoter. Neither a nonfunctional mutant of DAF-16 that fails to bind the KIX domain of CBP nor a nonresponsive homolog of DAF-16 that fails to bind SRC can inhibit glucocorticoid-responsive gene transcription. From this we conclude that, in the absence of insulin, a major role of DAF-16-like factors may be to enhance glucocorticoid stimulation of its target genes. We show that the DAF-16-like protein FKHR is most similar to DAF-16 in its ability to mediate the negative effect of insulin on transcription of the IGFBP-1 gene. Our findings suggest that insulin may alter the activity of certain DAF-16-like proteins by preventing their association with coactivator proteins in addition to promoting association with 14-3-3 and retention in the cytoplasm as previously proposed (18, 23, 24). Thus, dependence of certain glucocorticoid-responsive genes on an FKH accessory factor site would allow DAF-16-like proteins to integrate the opposing effects of glucocorticoids and insulin on specific target genes.

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